

Independence of dimethylamiloride-sensitive Li^+ efflux pathways and Na^+-Li^+ countertransport in human erythrocytes

Gianpaolo Zerbinì, Ruggero Mangili^{*}, Guido Pozza

Renal Pathophysiology Laboratory, Division of Medicine, Scientific Institute San Raffaele, University of Milan, Milan I-20132, Italy

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Abstract

The *in vivo* function of the erythrocyte Na^+-Li^+ countertransport (SLC) is unknown. Whether SLC may reflect an operational mode of the widespread Na^+-H^+ exchanger (NHE) or may otherwise be expression of an independent membrane transport, remains presently unclear. We explored the presence of 5-(*N,N*-dimethyl)-amiloride (DMA)-sensitive Li^+ pathways in human erythrocytes where the activity of the Na^+ pump, Na^+-K^+ cotransport and anion exchange were suitably inhibited. A total of 0.02 mM DMA had no effect on SLC as expected, but gave a significant inhibition of Li^+ efflux into both Na^+ and Na^+ -free media. This DMA-sensitive Li^+ pathway, but not SLC, was significantly enhanced by hyperosmolar cell shrinkage, which is a characteristic feature of NHE. In conclusion, DMA-sensitive Li^+ pathways, possibly mediated by NHE, are present in erythrocytes and coexist with the DMA-insensitive, SLC. This finding supports the notion that SLC is independent of amiloride-sensitive NHE. © 1998 Elsevier Science B.V.

Keywords: Na^+-Li^+ countertransport; Na^+-H^+ exchange; Amiloride; Erythrocyte; (Human)

1. Introduction

Erythrocyte Na^+-Li^+ countertransport (SLC) is a long-known function of the erythrocyte membrane in man and in several animal species [1,2], but its nature and role in physiology remain unclear. Na^+-H^+ exchanger (NHE) activity is ubiquitous in human tissues, where it can be mediated by one or more of

several isoforms [3–5], and is functionally detectable also in the erythrocyte [6–8], where only the amiloride-sensitive isoform NHE-1 appears to be detectable [9–11]. Studies of Na^+ flux across the membrane suggest that NHE may mediate a bidirectional 1:1 exchange of Li^+ for Na^+ or H^+ [12–15], which has contributed to the hypothesis that SLC may reflect an operational mode of this system [16,17]. Furthermore, abnormal elevations of the NHE activity are also found in essential hypertension [18–20] and in diabetic nephropathy [21–24]; these findings seem to parallel abnormally elevated SLC [22–25].

However, SLC seems totally insensitive to amiloride [26–29], and the finding that the inter-individual variability of SLC cannot be explained by polymorphisms of the gene encoding for NHE-1 [30] appeared to favour the alternative view that SLC may

Abbreviations: SLC, erythrocyte Na^+-Li^+ countertransport; NHE, Na^+-H^+ exchanger; DIDS, 4,4'-diisothiocyanato-stilbene-2,2'-disulfonic acid; DMA, 5-(*N,N*-dimethyl)-amiloride; MOPS, (morpholino)propane-sulfonic acid; BSA, bovine serum albumin

^{*} Corresponding author. Divisione Medicina I, Istituto Scientifico San Raffaele, Via Olgettina, 60, I-20132 Milano, Italy. Fax: +39-2-26433790; E-mail: mangili.ruggero@hsr.it

be mediated by a transmembrane carrier independent of amiloride-sensitive transports [27,30].

Reversible exchange of Na^+ or H^+ for $\text{Na}^+(\text{Li}^+)$ is an established property of NHE in several kinds of cells [12,13,31]. The erythrocyte seems, however, to lack a H^+ -driven $\text{Na}^+(\text{Li}^+)$ efflux, as external H^+ in this cell appears to inhibit $\text{Na}^+(\text{Li}^+)$ efflux [32,33], which is stimulated in other cells [12,13,31]. Interestingly, intracellular Li^+ can activate NHE even at resting intracellular pH [33], and an amiloride-sensitive Li^+ efflux pathway stimulated by hyperosmolarity has been detected in Li^+ loaded canine erythrocytes and attributed to NHE [33]. Functional studies aimed to detect the presence of amiloride-sensitive Li^+ pathways across the erythrocyte membrane have never been performed in humans.

Therefore, we explored the regulation of erythrocyte Li^+ efflux rates following challenge with specific inhibitors of SLC and NHE in isotonic media as well as in hyperosmolar solutions, which are known to enhance NHE [32,34,35] though not SLC activity [36].

2. Materials and methods

Eleven healthy members of Hospital Staff gave informed consent to participate in the study. A fasting blood samples was drawn from the antecubital vein from each individual and was processed within 2 h.

2.1. Reagents

Ouabain, bumetanide, 4,4'-diisothiocyanato-stilbene-2,2'-disulfonic acid (DIDS), 5-(*N,N*-dimethyl)-amiloride (DMA), nystatin, Me_2SO , Tris, (morpholino)propane-sulfonic acid (MOPS), bovine serum albumin (BSA), were purchased from Sigma Aldrich (Milan, Italy). LiCl monohydrate was purchased from Merck (Darmstadt, Germany). All other reagents and chemicals were purchased from BDH (Milan, Italy).

2.2. Erythrocyte Li efflux studies

Properties of erythrocyte Li^+ efflux were addressed following the original technique described by Canessa et al. [37] and Canessa [38], as subsequently modified by the use of nystatin to load cells with Li^+ [39]. In brief, after the removal of plasma and buffy

coat, washed erythrocytes were incubated for 20 min at 4°C in an unbuffered solution containing $40\text{ }\mu\text{g ml}^{-1}$ of nystatin, 50 mM sucrose, 130 mM KCl and 20 mM LiCl , followed by centrifugation and resuspension of the cell pellet for 10 min in the same solution, though without nystatin. The ionophore was removed by washing cells for four times at 37°C in a 1 mM K-phosphate buffer (pH 7.4) containing 50 mM sucrose, 130 mM KCl, 20 mM LiCl , 10 mM glucose and 1 mg l^{-1} BSA. External Li^+ was then removed by washing erythrocytes for four times at 4°C , in a neutral (pH 7.4) 10 mM Tris–MOPS buffer containing 150 mM choline–Cl and 1.0 mM MgCl_2 . The osmolarity of these solutions was always adjusted to range between 300 and 310 mosM. Li^+ -loaded erythrocytes were finally resuspended in cold washing buffer; aliquots from each individual were transferred into full set of efflux media, suitably matching a hematocrit of 3–4% in each efflux incubate.

Na^+ and choline (Na^+ -free) efflux media were isotonic and neutral (pH 7.4 at 37°C). These contained either 150 mM NaCl, or equimolar choline–Cl, in 10 mM Tris–MOPS buffer with 10 mM glucose and 1.0 mM MgCl_2 . Ouabain (0.1 mM), 0.1 mM bumetanide and 0.2 mM DIDS were always added to block, respectively, the activity of the Na^+ pump, $\text{Na}^+\text{--K}^+$ cotransport and anion exchanger [40–43].

To study Li^+ efflux, experiments were carried out in parallel incubates with or without the presence of 20 μM DMA and/or 200 μM phloretin.

To induce hyperosmotic shrinkage of erythrocytes, both efflux media were made hypertonic by adding 200 mM sucrose, to obtain a final osmolarity of 500–510 mosM. Under these conditions, the mean corpuscular volume can be reduced by $30 \pm 1.7\%$. To minimize the effect of inter-assay variability, erythrocytes from each individual were always tested with a full set of efflux media within a single experiment.

Duplicate aliquots of each incubate were taken at the beginning of the incubation and after 15 and 30 min at 37°C , were chilled in melting ice and centrifuged at 4°C . Supernatants were taken for the measurement of Li^+ concentrations by atomic absorption spectrophotometry (Perkin Elmer 4000).

Li^+ efflux rates were calculated in each medium as the change in Li^+ concentration over time by simple linear regression analysis, and expressed as

$\mu\text{mol l}_{\text{cell}}^{-1} \text{h}^{-1}$. The correlation coefficient was higher than 0.98 in all studies. As usual, SLC was taken as the difference between Li^+ efflux rates in Na^+ and choline media. The response of Li^+ efflux to hyperosmolar cell shrinkage and/or DMA was evaluated both in Na^+ and in choline media by calculating relevant differences in Li^+ efflux rates.

2.3. Statistical analysis

Data are shown as arithmetical mean with standard error. Comparisons between paired or observations were addressed by Student's *t*-test. Correlations were sought by simple linear regression. The null hypothesis was rejected for two-tailed *P* values less than 5%.

3. Results

SLC ranged between 164 and 783 $\mu\text{mol l}_{\text{cell}}^{-1} \text{h}^{-1}$ (mean \pm S.E.: 360 ± 53) in the 11 healthy individuals

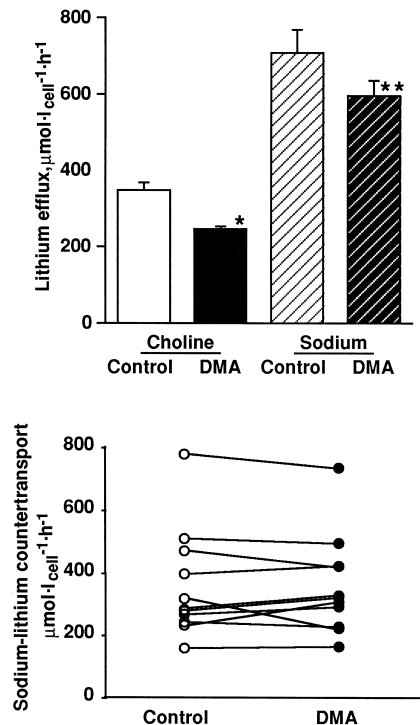


Fig. 1. Upper panel. Effect of DMA on Li^+ efflux from Li^+ -loaded erythrocytes into choline (* $P = 0.001$) or Na^+ media (** $P = 0.002$), $n = 11$. Lower panel. Effect of DMA on SLC activity ($P = \text{ns}$), $n = 11$.

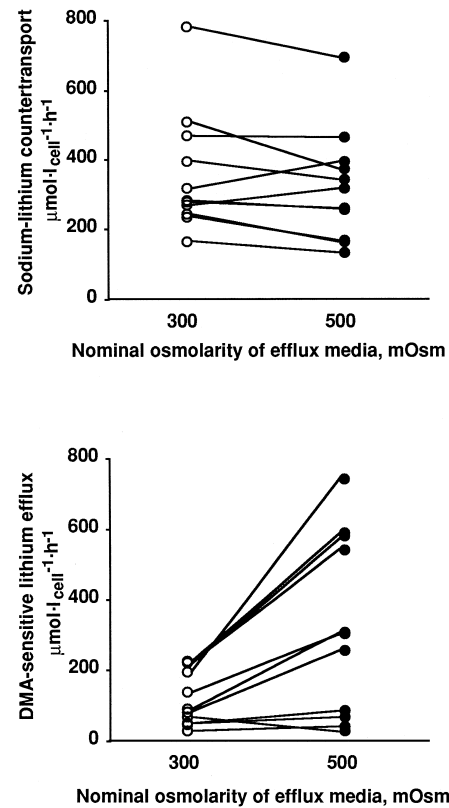


Fig. 2. Upper panel. Effect of hyperosmotic cell shrinkage on SLC activity ($P = \text{ns}$), $n = 11$. Lower panel. Effect of hyperosmotic cell shrinkage on the DMA-sensitive component of Li^+ efflux in choline medium ($P = 0.005$), $n = 11$.

considered for these studies. Li^+ efflux into Na^+ -free media, was $345 \pm 28 \mu\text{mol l}_{\text{cell}}^{-1} \text{h}^{-1}$ and was significantly inhibited to 243 ± 13 ($P = 0.001$) in the presence of DMA. However, total Li^+ efflux into Na^+ media was comparably inhibited from 705 ± 71 to $595 \pm 49 \mu\text{mol l}_{\text{cell}}^{-1} \text{h}^{-1}$ in the presence of DMA ($P = 0.002$), leaving SLC unchanged (Fig. 1). In line with these observations, DMA-sensitive components of Li^+ efflux in Na^+ media were highly correlated with those measured in Na^+ -free media ($R^2 = 0.83$, $P < 0.0001$), and standard SLC was likewise related with SLC as calculated in the presence of DMA ($R^2 = 0.92$, β -coefficient \pm S.E.: 1.07 ± 0.10 , $P < 0.0001$). The DMA sensitive component of Li^+ leak was unrelated with the complementary DMA-insensitive component ($R^2 = 0.16$, $P = \text{ns}$); there was a weak positive association with SLC that missed statistical significance ($R^2 = 0.31$, $P = 0.07$). Phloretin

significantly inhibited SLC (from 360 ± 53 to $64.7 \pm 12 \mu\text{mol l}_{\text{cell}}^{-1} \text{h}^{-1}$, $P < 0.0005$).

Hyperosmolar shrinkage of erythrocytes induced significant but parallel increases in Li^+ efflux both into Na^+ (from 705 ± 71 to $995 \pm 122 \mu\text{mol l}_{\text{cell}}^{-1} \text{h}^{-1}$, $P = 0.0011$) and into choline media (from 345 ± 28 to $668 \pm 81 \mu\text{mol l}_{\text{cell}}^{-1} \text{h}^{-1}$, $P = 0.0002$), leaving again SLC unchanged (327 ± 48 vs. standard SLC, $P = \text{ns}$) and strongly correlated with standard SLC ($R^2 = 0.87$, β -coefficient $\pm \text{S.E.} = 1.01 \pm 0.12$, $P < 0.0001$). The DMA-sensitive component in Li^+ efflux was increased by hyperosmotic shrinkage, both in choline (from 110 ± 21 to $282 \pm 67 \mu\text{mol l}_{\text{cell}}^{-1} \text{h}^{-1}$, $P = 0.005$, Fig. 2) and in Na^+ media (from 109 ± 31 to 328 ± 26 , $P = 0.003$). Phloretin inhibited the SLC also after hyperosmotic shrinkage (from 327 ± 48 to $48.0 \pm 12 \mu\text{mol l}_{\text{cell}}^{-1} \text{h}^{-1}$, $P < 0.0005$).

4. Discussion

The present study extends the concept that SLC is entirely insensitive to amiloride in human erythrocytes [26–29], but can be largely inhibited by phloretin in all individuals [2,26,44]. Furthermore, we describe for the first time in human erythrocytes a SLC-independent, DMA-sensitive component of Li^+ efflux that can be activated by hyperosmolar cell shrinkage, which is a known stimulus of NHE [32,34,35]. Evidence that such a component of Li^+ efflux may be mediated by NHE has not been directly provided by the present study, but the demonstration of DMA-sensitivity and the possibility to enhance its magnitude by hyperosmolar cell shrinkage is consistent with this view. In contrast, SLC failed to increase its activity under hyperosmolar conditions, in line with previous evidence [36].

While emphasizing the independence of SLC from amiloride-sensitive fluxes, the possibility that amiloride-insensitive and as yet undefined isoforms of NHE may be present in erythrocytes cannot be entirely ruled out. Amiloride-insensitive NHE has been reported in other cells [45–47] raising the possibility that SLC maybe mediated by a similar carrier. However, while sensitivity to phloretin is an invariable feature of SLC [2,26,44], this property does not characterize the amiloride-insensitive NHE of human thymic lymphocytes [45]. A second amiloride-insensi-

tive NHE was described in rat hippocampal synaptosome preparations and could be inhibited by harmaline [46], that fails however to inhibit SLC in our laboratory (unpublished observation). Taken together, these observations further reduce the possibility that SLC may reflect an operational mode of amiloride-sensitive NHE isoforms, leaving its nature and role in physiology open to speculation. It has been suggested that SLC operates a futile $\text{Na}^+ - \text{Na}^+$ exchange in vivo, but the presence of such an exchange is controversial in human erythrocytes [48,49]. A third possibility is that SLC may just represent a degraded remnant with no physiologic role in the circulating mature cell.

Based on the finding of parallel differences in NHE and SLC activity rates in essential hypertension and diabetic nephropathy, a correlation between the V_{max} of NHE and that of SLC has been suggested in erythrocytes [25,50]. This may be in line with the sharing of one or more regulatory pathways, although this correlation was not found by other authors [19].

Our suggestion that SLC is independent of amiloride-sensitive membrane transports appears to encourage a better definition of the mechanisms that underlie its elevated rates in human disease [51–54] and the search for a candidate SLC gene in essential hypertension [55] and/or diabetic nephropathy [56].

Acknowledgements

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